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Chronic impacts of invasive herbivores on a foundational forest species: a whole-tree perspective

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1 **Running head:** Herbivore impacts on tree health

2

3 **Title:** Chronic impacts of invasive herbivores on a foundational forest species: a whole-
4 tree perspective

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24 **Abstract**

25 Forests make up a large portion of terrestrial plant biomass, and the long-lived woody
26 plants that dominate them possess an array of traits that deter consumption by forest pests.
27 Although often extremely effective against native consumers, invasive species that avoid or
28 overcome these defenses can wreak havoc on trees and surrounding ecosystems. This is
29 especially true when multiple invasive species co-occur, since interactions between invasive
30 herbivores may yield non-additive effects on the host. While the threat posed by invasive forest
31 pests is well known, long-term field experiments are necessary to explore these consumer-host
32 interactions at appropriate spatial and temporal scales. Moreover, it is important to measure
33 multiple variables to get a 'whole-plant' picture of their combined impact. We report the results
34 of a four-year field experiment addressing the individual and combined impacts of two invasive
35 herbivores, the hemlock woolly adelgid (*Adelges tsugae*) and elongate hemlock scale (*Fiorinia*
36 *externa*), on native eastern hemlock (*Tsuga canadensis*) in southern New England. In 2011, we
37 planted 200 hemlock saplings into a temperate forest understory and experimentally manipulated
38 the presence/absence of both herbivore species; in 2015, we harvested the 88 remaining saplings
39 and assessed plant physiology, growth, and resource allocation. Adelgids strongly affected
40 hemlock growth: infested saplings had lower above/belowground biomass ratios, more needle
41 loss, and produced fewer new needles than control saplings. Hemlock scale did not alter plant
42 biomass allocation or growth, and its co-occurrence did not alter the impact of adelgid. While
43 both adelgid and scale impacted the concentrations of primary metabolites, adelgid effects were
44 more pronounced. Adelgid feeding simultaneously increased free amino acids local to feeding
45 sites and a ~30% reduction in starch. The cumulative impact of adelgid-induced needle loss,
46 manipulation of nitrogen pools, and the loss of stored resources likely accelerates host decline

47 through disruption of homeostatic source-sink dynamics occurring at the whole-plant level. Our
48 research stresses the importance of considering long-term impacts to predict how plants will
49 cope with contemporary pressures experienced in disturbed forests.

50

51 *Keywords:* exotic species invasions, forest understory, herbivory, piercing-sucking
52 insects, plant resource allocation, plant primary metabolism, *Tsuga canadensis*

53

54 **Introduction**

55 Forests make up a large fraction of terrestrial plant biomass and provide a wide variety of
56 ecologically- and economically-important ecosystem functions. The long-lived woody plants that
57 dominate these systems possess a formidable array of both constitutive and inducible defenses
58 against exploitation (Coley et al. 1985). While plant-consumer coevolution selects for defenses
59 effective against native exploiters, it may not protect against newly-arrived consumers with
60 novel feeding modes or attack strategies. In such situations, the mismatch in generation time
61 between long-lived woody plants and their consumers may prove catastrophic: invasive species
62 have driven multiple tree species to functional extinction (Boyd et al. 2013).

63 The threat posed by invasive species is particularly acute in temperate forests (Lovett et
64 al. 2006, Gandhi and Herms 2010). These regions have relatively low family-level woody plant
65 diversity and are dominated by a small number of tree species. In addition, global transportation
66 networks linking formerly-disjunct temperate regions have sharply increased the potential for,
67 and number of, species invasions (Lovett et al. 2006, Gandhi and Herms 2010). As a result,
68 many temperate tree species are now forced to contend with multiple invasive species as well as
69 their native consumers. The cumulative impact of multiple herbivores is rarely additive, with the

70 outcome often depending on the sequence of attack (Ali and Agrawal 2014) and the feeding
71 guild of the insect (Zvereva et al. 2010). Such non-additive effects are particularly likely when
72 early-arriving herbivores induce changes in the host plant (Fournier et al. 2006, Morris et al.
73 2007, Pieterse and Dicke 2007, Stam et al. 2014) that alter the impact of later-arriving species
74 (Wallin and Raffa 2001, Soler et al. 2012).

75 There are two key mechanisms by which herbivores impact plants. They can alter
76 performance traits (growth, reproduction and survival) of the host and/or they can induce local
77 and systemic changes in plant chemistry. Both mechanisms may affect the susceptibility,
78 resistance or tolerance of plants to subsequent attack and can mediate subsequent interactions
79 among herbivores (Denno et al. 1995, van Zandt and Agrawal 2004, Viswanathan et al. 2005).
80 For example, reductions in foliar nutrients or changes in defensive chemistry following damage
81 are well known to affect the suitability of hosts for late-arriving herbivores, with consequences
82 for growth and survival (McClure 1980, Inbar et al. 1999, Soler et al. 2007). These changes may
83 magnify the impact of one or both herbivores, leading to invasional meltdown (Simberloff and
84 Von Holle 1999); alternately, they can decrease the cumulative impact and generate invasional
85 interference (Yang et al. 2011, Rauschert and Shea 2012). Understanding what factors determine
86 the outcome of herbivore interactions on a shared host is especially important for sap-feeders, a
87 group whose impact on plant fitness can equal or exceed that of defoliators (Zvereva et al. 2010).
88 Given these plant-wide effects, a 'whole-plant' analysis of herbivore-induced changes is required.

89 Work addressing forest pest invasions generally takes one of two approaches. Examining
90 pests at the forest scale provides important data on long-term trends in plant health and pest
91 densities, but the logistical constraints inherent in such large-scale and long-term research means
92 that such work is rarely experimental (Preisser et al. 2008). This is important since studies

93 comparing naturally-infested and herbivore-free trees in order to assess herbivore impacts (e.g.,
94 Domec et al. 2013) conflate cause and effect and cannot be used to quantify non-additive effects
95 (Nykänen and Koricheva 2004). Conversely, efforts addressing the impact of pests on plant
96 physiology or chemistry are often short-term (i.e., <1 year in duration) and examine a subset of
97 plant traits. The latter type of study are also often conducted in relatively controlled settings
98 (e.g., greenhouses or plantations) whose abiotic conditions may differ markedly from natural
99 systems (e.g., Miller-Pierce et al. 2010). While great strides have been made using both
100 approaches, understanding some aspects of forest invasions may require *in situ* field experiments
101 that are conducted at system-appropriate temporal/spatial scales and measure a wide array of
102 plant traits in order to produce a 'whole-organism' picture.

103 Regardless of approach, relatively little work on forest pests has addressed their impact
104 on the ontogenetic stages necessary for stand regeneration and succession. Because seedlings and
105 saplings can live for decades in the low-light forest understory, their responses to herbivory may
106 not match those of mature trees (Boege and Marquis 2005, Barton and Koricheva 2010). For
107 example, understory saplings that rely on early-spring carbon acquisition prior to canopy leaf-out
108 (Hadley and Schedlbauer 2002, Polgar and Primack 2011) may be especially harmed by
109 decreased photosynthesis following attack. Such impacts may influence resource allocation
110 trade-offs and alter plant functional priorities concerning growth, resource acquisition and
111 herbivore defense (Boege and Marquis 2005).

112 We aim to examine the complex ways in which multiple herbivores impact the
113 physiology and growth of a long lived woody plant. In order to address this, we utilize a large-
114 scale and long-term field experiment. This unique design examines the individual and combined
115 impacts of two invasive herbivores, the hemlock woolly adelgid (*Adelges tsugae*) and elongate

116 hemlock scale (*Fiorina externa*), on the growth, physiology and chemistry of eastern hemlock
117 (*Tsuga canadensis*, 'hemlock') understory saplings. The two pests co-occur in a portion of their
118 ranges – especially in southern New England, New York, and Pennsylvania. This co-occurrence
119 has become more pronounced over the past three decades as the ranges have shifted (see
120 appendix S1, 'Natural History of the System', for additional details). In 2011, we planted several
121 hundred hemlocks into a deciduous forest understory in southern New England (USA) and
122 inoculated them individually, simultaneously, or sequentially with one, both, or neither herbivore
123 over a four-year period. In 2015, we harvested the hemlocks and quantified multiple aspects of
124 growth, metabolism, and resource allocation in both above- and below-ground tissue. Our
125 'whole-tree' results reveal the disparate impact of these two herbivores and the complex ways in
126 which herbivory alters woody plant growth and physiology.

127

128

129 **Materials and Methods**

130 In April 2011, 200 ~0.3 m tall hemlock saplings (Van Pines Nursery, West Olive, MI,
131 USA) were planted into a hardwood (maple/oak dominated) forest at the Kingston Wildlife
132 Research Station (Kingston, RI). The trees had not been treated with insecticide. Saplings were
133 planted in a 10 x 20 grid ~1.25 m from each other; initial heights and basal diameters were
134 recorded prior to planting. Each sapling was enclosed in a mesh-covered (Agribon-15, Johnny's
135 Selected Seeds, Waterville, ME, USA; 90% light) wire cage to exclude deer browsing and
136 prevent cross-treatment contamination. The mesh bags were removed between December and
137 March, while both insects are immobile, to prevent snow from collapsing the cages.

138 Following planting, each tree was randomly assigned to an herbivore treatment (Table 1).

139 Inter-plant adelgid and scale dispersal is most likely to occur prior to spring leaf-out, when both
140 sub-canopy wind velocities and crawler densities are high (McClure 1989). Each spring, we
141 simulated yearly dispersal by inoculating each tree with foliage infested with the appropriate
142 insect; herbivore-free trees were 'inoculated' with uninfested foliage. Herbivore-infested foliage
143 was collected from singly-infested stands previously identified in surveys (Gómez et al. 2015).
144 Inoculations were conducted using a standard protocol (Butin et al. 2007); because adelgid
145 emerges earlier than EHS, inoculations were conducted in May and June, respectively.

146 Starting in 2011, trees in three treatments were annually inoculated with adelgid ('HWA')
147 only, scale ('EHS') only, both, or neither (=uninfested foliage) insects for four years (HWA-4,
148 EHS-4, and Both-4, respectively). Starting in 2013, some adelgid-only and some scale-only trees
149 were thereafter annually inoculated with both insects, creating two 'priority effect' treatments
150 (HWA→Both, EHS→Both). In 2013, we also began annual inoculations of previously-
151 uninfested trees with adelgid-only, scale-only, or both insects for two years (HWA-2, EHS-2,
152 Both-2). A subset of trees remained herbivore-free throughout (Control; Table 1).

153 Insect densities were assessed twice yearly, in early spring and late fall, throughout the
154 experiment. Details regarding insect densities from 2011-2014 are presented elsewhere
155 (Schaeffer et al. 2017). Because our focus is on cumulative treatment impacts, we report
156 November 2014 insect densities solely as an indicator of whole-tree infestation levels. In fall
157 2014, insect densities on newly-produced foliage were similar for adelgid (2.01 ± 0.18 [SE]
158 insects cm^{-1}) and scale (1.99 ± 0.26 insects cm^{-1}) (Table 1). These infestation levels fall within
159 those observed in the field and in prior studies where hemlock trees were experimentally
160 inoculated (Miller-Pierce et al. 2010, Soltis et al. 2015). As in prior work, the densities of both
161 adelgid and scale were higher in single-species treatments than when they co-occurred (150%

162 higher for adelgid and 50% higher for scale), suggesting plant-mediated interference competition
163 between these two herbivores (Preisser and Elkinton 2008).

164 Between 2011-2015, we lost replicates to Hurricane Sandy, cross-treatment
165 contamination, browsing by white-tailed deer (*Odocoileus virginianus*), and isolated outbreaks of
166 secondary pests (e.g., *Oligonychus ununguis* mites and *Nucalaspis* sp. scales). There were
167 several trees in the single-herbivore treatments (i.e., treatments EHS-2, EHS-4, HWA-2, and
168 HWA-4) whose low insect densities (<0.5 insects/cm; the bottom 15% of fall 2014 densities)
169 may have obscured the impact of insect damage; we excluded these trees from the harvest. The
170 88 remaining trees were intensively monitored in early spring prior to the May 2015 harvest.

171 *Spring monitoring and harvest.* In early April 2015, three branches per tree were selected
172 and marked. Between 15-19 April (prior to bud break and crawler emergence), these branches
173 were used to quantify herbivore abundance and photosynthetic rates. Because insects were
174 located on older needles, we calculated insect densities per marked branch by dividing the
175 number of insects by ≥ 1 -year needle biomass (insects g^{-1} DW). We chose this metric because (1)
176 adelgid settles at the needle base while scale settles on the needles; and (2) similarly-sized
177 branches could vary in needle density (C. Wilson, *personal observation*). As a result, expressing
178 density on a per-gram basis provided a more ecologically-relevant density metric in this case.

179 Photosynthetic rates were measured between 0800 and 1200 using one-year-old (2014
180 growth) foliage on the terminal end of each marked branch using a CIRAS-2 portable
181 photosynthesis system (PP systems, Haverhill, MA, USA) with a 2.5 cm^2 cuvette and a CIRAS-2
182 LED light source of $1,500 \mu\text{mol m}^{-2} \text{ s}^{-1}$, a CO_2 concentration of 390 ppm, air flow rate at 350 cm^3
183 s^{-1} , and leaf temperature of 25°C . After each measurement, the foliage was photographed and
184 ImageJ 1.44 (Abramoff et al. 2004) used to quantify needle area.

185 The 88 experimental trees were harvested over a 14-day period in May 2015. The time
186 and effort required for whole-tree excavation required us to split the trees into 22 four-tree
187 harvest groups, with each treatment represented in at least every third group; 1-3 groups were
188 harvested daily. Data on the timing of bud break is presented elsewhere, along with similar data
189 from another multi-year experiment (Whitney et al in preparation); bud break data in this paper
190 is used solely to calculate new flush production.

191 *Whole-plant biomass distribution.* Immediately prior to harvesting each tree, we recorded
192 its height and trunk diameter five cm above the root ball. Each marked branch was then clipped
193 at the base and placed on ice in a plastic bag. To ensure that we obtained a sufficient amount of
194 plant material for chemical analyses, we collected an additional randomly-selected branch from
195 each tree; all four branches were immediately transported to the laboratory for processing
196 (detailed below). The trunk of each tree was then clipped five cm above the root ball and the
197 aboveground portion dried for 24 hrs at 60° C. We sorted dry material into three classes (new
198 flush, \geq 1-yr needles, and wood). After the aboveground portion of each tree had been removed,
199 its root ball was excavated, cleaned of all dirt and foreign objects, dried as above, and weighed.
200 Belowground harvest and processing protocols are detailed elsewhere (Schaeffer et al. 2017).

201 *Chemical analyses.* In the laboratory, all insects on marked branches were removed using
202 a dissecting scope to avoid damaging any hemlock tissue. Each branch was separated into five
203 tissue types (new flush, 1-yr old needles, >1-yr old needles, 1-yr old stems, and >1-yr old stems)
204 and weighed; the fresh mass of each tissue was converted to dry mass using tissue-type-specific
205 conversion factors generated in a pilot experiment (Appendix S2: Table S1). Each type was kept
206 separate for each tree, stored at -20°C before being dried at -55°C for 72 hrs in a lyophilizer, then
207 ground into powder using a KLECO ball mill (Garcia Machines, Visalia, CA, USA).

208 Carbon (C) and nitrogen (N) content were determined by dry-combusting 2–3 mg of
209 finely-ground material with a CHNOS analyzer (vario Micro cube, Elementar Americas, Mt.
210 Laurel, NJ, USA). Starch was quantified using an EnzyChrom™ starch assay kit (BioAssay
211 Systems, Hayward, CA, USA) as per manufacturer protocols. Briefly, 10 mg of root powder was
212 boiled in one mL distilled water for five min, then centrifuged at 10,000 x g for two min; the
213 supernatant containing soluble starch was set aside. The remaining pellet was reconstituted in 0.2
214 mL dimethyl sulfoxide and boiled for five min to obtain recalcitrant starch. The supernatants
215 were combined and tissue starch levels (mg g^{-1} DW) determined using the kit.

216 We quantified free tissue amino acid levels and relative composition of individual amino
217 acids following the protocol of Gomez et al. (2012). For each needle tissue type, 0.2 g of sample
218 material was extracted in one mL 80% ethanol (v:v) at room temperature for 20 min. Samples
219 were vortexed periodically and then centrifuged at 10,000 x g for ten min at room temperature.
220 The supernatant was filtered through a 0.45 μm Acrodisk Syringe filter (Pall Gelman Laboratory,
221 Ann Arbor, MI, USA), and the filtrate used for free amino acid determination using a
222 commercial EZ: Faast™ kit (Phenomenex, Torrence, CA, USA) and GC-FID (Agilent
223 Technologies, Waldbron, Germany) as per manufacturer protocols. Briefly, two μL of sample
224 was injected (15:1 split) on a Zebron ZB-AAA column (0.25 mm x 10 m; Phenomenex) with the
225 injector temperature set to 250°C. Helium was used as the carrier gas at a flow rate of 1.5 mL
226 min^{-1} . The initial oven temperature was set to 110°C, increased at a linear rate of 32° min^{-1} to
227 320°C, and held at 320°C for three min. This kit identifies and quantifies 22 amino acids;
228 individual amino acids were identified by comparing retention times to amino acid standard
229 solutions (norvaline as internal standard) and quantified using ChemStation software (Rev.
230 B.04.02; Agilent Technologies, Waldbron, Germany).

231 *Statistical analyses.* All analyses were performed using R v. 3.2.2 (RCoreTeam 2014).
232 Welch's *t*-tests were used to compare insect densities. We fit linear mixed-effects models and
233 used a backward-model-selection approach to examine how the individual and interactive effects
234 of adelgid and scale on hemlock. Adelgid and scale were treated as fixed factors, each with three
235 levels corresponding to the length of infestation (0, 2, or 4 years) and an interactive term
236 (HWA*EHS). Full and reduced models were ranked and compared based on Bayesian
237 Information Criterion (BIC) values, a standard criterion for model selection. Details of each
238 model, including the random effects used for each, are contained in Appendix S3. The *lme4*
239 package was used to generate and compare models (Pinheiro et al. 2014). We used this approach
240 to examine the individual and combined impact of adelgid and scale, as well as how herbivore-
241 specific priority effects, affected the following: final height, final basal diameter, total biomass,
242 aboveground biomass, belowground biomass, above-/belowground biomass ratio, needle/woody
243 biomass ratio, new flush production, and photosynthesis.

244 Because tissue type and age can impact plant chemistry, we analyzed percent C, percent
245 N, C:N ratio, total amino acids, and total starch using a modified approach. For stem and needle
246 tissue, tissue age (1-year or >1-year) was included in the models. Because we did not have
247 enough 1-year tissue to conduct a full suite of chemical analyses on it, our analyses of 1-year
248 tissue are limited to percent C, percent N, and C:N ratio. We ran linear mixed-effects models
249 with row and harvest date as random effects.

250 For amino acid analyses, 1-year and > 1-year needles were analyzed separately. To assess
251 the effect of adelgid on amino acid levels, individual amino acids that were detected in <20% of
252 all biological replicates and constituted <1% of the total amino acids ($\mu\text{g g}^{-1}$ DW) were removed
253 from the datasets in order to prevent their over-influence in the analysis of profiles. The detection

254 of these amino acids followed no pattern with regards to treatment effects (logistic regressions; P
255 > 0.05). For 1-year needles, these were: alpha-aminobutyric acid (ABA; detected in 3%), beta-
256 aminoisobutyric acid (BAiB; detected in 19%), ornithine (ORN; detected in 13%), and sarcosine
257 (SAR; detected in 2%), and for > 1 -year needles, these amino acids were: alpha-aminobutyric
258 acid (ABA; detected in 9%), ornithine (ORN; detected in 5%), and sarcosine (SAR; detected in
259 10%). For the remaining amino acids, tissue levels ($\mu\text{g g}^{-1}$ DW) were Hellinger-transformed to
260 normalize data on a total μg amino acid basis; transformed values were used in profile analyses.

261 Treatment differences in amino acid profiles were visualized with NMDS using the
262 'Bray-Curtis' dissimilarity index and the 'vegan' package (Oksanen et al. 2013) in R. This index
263 was chosen because it consistently gave the highest rank-order similarity of all possible
264 dissimilarity indices available in the 'vegan' package that account for amino acid abundance, and
265 fitted the NMDS model with the lowest stress statistic (< 0.2 for all ordinations). The effect of
266 adelgid, scale, and their interaction on needle amino acid profiles was assessed via
267 PERMANOVA in the 'vegan' package with 10,000 permutations (Lieurance et al. 2015).

268 The effect of adelgid and scale on total amino acids was assessed via ANOVA followed
269 by a post-hoc Tukey test. The influence of adelgid and scale infestation on individual amino
270 acids was evaluated by first normalizing μg amino acid per mg total amino acids per g dry tissue
271 mass ($\mu\text{g mg}^{-1} \text{g}^{-1}$ DW), and then fitting an ANOVA model with adelgid infestation as the
272 predictor; scale and the HWA*EHS interaction were removed from all regressions because
273 neither influenced amino acid levels. The Benjamini-Hochberg false discovery rate-controlling
274 procedure (Benjamini and Hochberg 1995) was used to correct for multiple comparisons.

275 **Results**

276 *Growth and biomass allocation.* Adelgids altered hemlock growth (i.e., final

277 measurements with initial values, when significant, present as a covariate) and biomass
 278 allocation; scales did not. There were no significant priority effects (i.e., prior colonization by
 279 one species did not affect the impact of the later-arriving species), and the HWA*EHS
 280 interaction was never significant. Because of this, we report only the main insect effects in the
 281 text (see Appendix S3 for full model outputs). Although neither insect affected total, above-, or
 282 below-ground plant biomass, adelgid altered plant biomass allocation (Fig. 1; Appendix S3:
 283 Tables S1 and S2). The above-/below-ground biomass ratio of adelgid-infested trees was 17%
 284 lower than adelgid-free trees ($F_{2,79}=6.62, P=0.01$; Fig. 1a) and the aboveground needle/woody
 285 biomass ratio was 16% lower in adelgid-infested trees ($F_{2,78}=4.53, P=0.01$; Fig. 1c). Adelgid-
 286 infested trees were also 7% shorter than adelgid-free trees ($F_{2,78}=3.67, P=0.03$), but did not
 287 differ in final basal diameter (Appendix S3: Table S1).

288 *Early spring growth and photosynthesis.* New flush production (grams/day) prior to
 289 harvest was ~30% lower for adelgid-infested versus adelgid-free trees ($F_{2,77}=36.54, P<0.001$;
 290 Fig. 2a), but was not reduced by scale ($F_{2,77}=1.90, P=0.16$; Fig. 2b).

291 There were no significant treatment-level effects of adelgid or scale on photosynthetic
 292 rates of 1-year-old foliage (Fig. 3a; Appendix S3: Table S3). Although there was no relationship
 293 between adelgid density and photosynthetic rates (Appendix S3: Table S4), scale density was
 294 negatively correlated with photosynthetic rates in trees infested with scale for two and four years
 295 (Appendix S3: Table S5).

296 *Foliar chemistry.* While adelgid substantially altered multiple aspects of foliar chemistry,
 297 scale had no significant impact. The N content of 1-year needles in adelgid-infested trees was
 298 10% higher than in adelgid-free trees ($F_{2,166} = 10.80, P < 0.001$; Fig. 3b; Appendix S3: Table
 299 S7). Among trees infested with adelgid for two years, adelgid density was correlated with

300 percent N; this was not, however, the case among trees infested for four years with adelgid
301 (Appendix S3: Table S4). New-flush needles on adelgid -infested trees also had higher N levels
302 ($F_{2,69} = 4.22$, $P = 0.02$; Fig. 3b). Although starch concentration in 1-year needles was similar in
303 adelgid-free trees and trees infested with adelgid for two years, the starch content of trees
304 infested with adelgid for four was ~30% less than that of the other treatments (Fig. 3c). Scale
305 feeding increased starch in 1-year needles ($F_{2,155} = 3.95$, $P = 0.02$; Appendix S3: Table S8),
306 although total starch concentration was correlated with neither adelgid nor scale density
307 (Appendix S3: Tables S4 and S5).

308 Adelgid infestation altered the amino acid profiles (PERMANOVA; $P < 0.0001$) of both
309 1- and >1-year needles (Fig. 4a, 1-year needles; Fig. 4b, >1-year needles), while scale did not
310 (PERMANOVA; $P > 0.80$ for both). Valine, proline, isoleucine, and tryptophan were the major
311 drivers for both tissue types (Table 2a and 2b). Total free amino acid levels were greater in
312 adelgid-infested foliage for both 1-year (ANOVA; $F_{2,83} = 7.87$, $P < 0.001$; Table 2a) and >1-year
313 needles (ANOVA; $F_{2,83} = 11.90$, $P < 0.001$; Table 2b) vs. non-infested trees, a difference driven
314 primarily by proline. Two- and four-year infested foliage were not, however, significantly
315 different (post-hoc Tukey HSD).

316 Discussion

317 Exotic insects are an imposing force on native trees and their associated communities
318 (Lovett et al. 2006). Despite this, and the fact that native hosts often face attack by multiple
319 exotic herbivores (Gandhi and Herms 2010), *in situ* experimental evidence of the overall
320 consequences of attack for susceptible native species remains rare (Preisser et al. 2008). Our
321 multi-year manipulative study on woody plants growing in a natural setting provides a 'whole-
322 plant' perspective on how multiple invasive herbivores affect the growth and chemistry of a

323 naïve native tree. We found that chronic herbivory by two invasive piercing-sucking herbivores
324 had divergent impacts on the growth and chemistry of their shared host, a foundational tree
325 species in the temperate forests of the eastern United States (Ellison et al. 2005).

326 While multiple years of adelgid herbivory altered patterns of biomass allocation and
327 primary metabolism in understory hemlock saplings, scale had minimal impacts. Although
328 adelgid densities were lower when they co-occurred with scale ($t= 46.93$, $P < 0.01$; Table 1),
329 neither prior nor simultaneous inoculation of hemlock saplings with scale altered the impact of
330 adelgid on these understory plants. In general, dually-infested trees showed changes in allocation
331 and metabolites typical of adelgid-only treatments. One possible explanation for the subdued
332 effect of the scale is the presence of native armored scale (*Abgrallaspis ithacae*) on eastern
333 hemlock. No native adelgid species attacks eastern hemlock. Since the introduction of the
334 elongate hemlock scale did not present an entirely novel challenge to the host, the host may
335 already have some existing defenses.

336 *Plant growth and biomass distribution.* Several years of adelgid infestation on hemlock
337 saplings lowered above-/belowground and needle/woody tissue ratios. This is consistent with
338 previous work (Soltis et al. 2014, Soltis et al. 2015), and was likely driven by a combination of
339 reduced new foliar growth and premature needle desiccation/ loss. Our findings contrast with
340 research on other plant species that respond to aboveground herbivory by shifting resources
341 away from herbivore feeding sites and into stem and root storage sites (Babst et al. 2005, Babst
342 et al. 2008). Despite adelgid-infested and adelgid-free trees having similar per-needle
343 photosynthetic rates, the reduced production of new foliage, likely in combination with the loss
344 of old needles, clearly hampers resource uptake in a light-limited environment. In turn, this
345 restriction affected the production and allocation of primary metabolites in stems and needles.

346 *Primary metabolites.* Adelgid impacts on hemlock health were further reflected through
347 changes in primary metabolites. Herbivore-attacked plants often protect themselves via induced
348 changes in primary and secondary metabolism (Stam et al. 2014, Zhou et al. 2015), although
349 research to date has primarily addressed impacts of herbivory on secondary rather than primary
350 metabolism (Zhou et al. 2015). Our results also confirm previous work (Gómez et al. 2012)
351 showing that adelgids cause localized increases of N and the amino acid proline at their feeding
352 sites. Proline accumulation is a common plant response to drought stress (Delauney and Verma
353 1993); this and other adelgid-induced changes in hemlock physiology (Radville et al. 2011,
354 Domec et al. 2013) suggest that adelgid likely induces drought-like stress in its native host plant
355 (Gómez et al. 2012). For instance, paralleling increases in proline, adelgid-infested tissues had
356 lower levels of the amino acids isoleucine and tryptophan. A similar pattern has been observed in
357 *Arabidopsis* following aphid feeding. In *Arabidopsis* this pattern is associated with aphid-
358 induced increases in the hormone abscisic acid (ABA; Hillwig et al. 2016): adelgid also induces
359 ABA production following attack (Schaeffer et al. 2017). Although ABA induction is often
360 associated with water stress (Lee and Luan 2012), its induction may benefit piercing-sucking
361 insects via its antagonistic interactions with jasmonic acid (JA) signaling (Erb et al. 2009, Vos et
362 al. 2013), a key pathway for anti-herbivore defense. We hypothesize that ABA induction
363 following adelgid feeding aids its success through prevention of effective JA pathway signaling,
364 which is known to deter HWA crawlers (Schaeffer et al. 2017)

365 Starch is another key primary metabolite which plays an essential role in plant tolerance
366 to damage. Following herbivory, stored carbohydrates are frequently broken down and
367 remobilized to compensate for tissue loss (Appel et al. 2014). The post-attack mobilization of
368 these resources can benefit the host by fueling repair and regrowth (Trumble et al. 1993). Some

369 herbivores, particularly piercing-sucking insects, exploit hosts and stored resources via extra-oral
370 digestion of stored carbohydrates like starch. This extra-oral digestion is achieved via
371 deployment of salivary enzymes like α -amylase to local feeding sites. Adelgid, a piercing-
372 sucking herbivore, has been hypothesized to use a similar feeding strategy (Oten et al. 2014).
373 Our findings support this hypothesis: adelgid feeding for four years led to a ~30% reduction in
374 starch levels in 1-year needles (Fig. 3c). The loss of stored resources through feeding, combined
375 with loss of source tissues, likely accelerates host decline through disruption of homeostatic
376 source-sink dynamics occurring at the whole-plant level.

377 *Perspective on the impacts of multiple invasive herbivores across space and time.* Plant
378 stresses, especially when experienced during early ontogenetic stages, strongly affect resource
379 allocation trade-offs concerning growth, resistance, storage, and reproduction (Boege and
380 Marquis 2005). Understanding such trade-offs requires studies conducted at the appropriate
381 temporal and spatial scales. Despite the lack of interference between adelgid and scale on any
382 metric of hemlock health in this experiment, our observation of suppressed adelgid densities
383 when co-occurring with scale (Table 1; also see Schaeffer et al. 2017), combined with multiple
384 years of landscape-level observations (Gómez et al. 2015), suggests that the impact of scale on
385 adelgid may be density-dependent and will likely become more pronounced on the landscape
386 over time. Prior work in this system has found that higher densities of scale can significantly
387 reduce adelgid densities and benefit the native host (Preisser and Elkinton 2008). Moreover,
388 while adelgid densities have generally declined in our region of study over time, scale densities
389 have steadily increased, effectively making scale the most abundant hemlock herbivore
390 throughout much of New England (Gomez et al. 2015, Schliep et al. 2018). As scale abundance
391 continues to increase, we predict that interference competition between these two herbivores

392 could buffer future declines of this foundational forest species in southern New England – where
393 the ranges of the two pests overlap most prominently. While this may facilitate hemlock
394 recovery in the northern portion of the invaded range, the impact on hemlock decline in the mid-
395 Atlantic portion of the United States requires further study. Less certain, however, is how the two
396 pests will interact with the shifting range of their host (McAvoy et al. 2017, Rogers et al. 2017).

397 In conclusion, we found that two invasive herbivores from the same feeding guild have
398 disparate effects on biomass allocation, growth, and primary metabolism of an early ontogenetic
399 stage of a foundational forest species. Our research stresses the importance of considering long-
400 term impacts for predicting woody plant responses to contemporary pressures experienced in
401 disturbed forests, especially in the case of life-stages that will dictate their future prosperity.

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- 570

571 **Table 1:** Treatments are arranged in a 3 x 3 full-factorial design, with years of infestation
 572 by both hemlock woolly adelgid (HWA) and elongate hemlock scale (EHS) indicated. Numbers
 573 in parentheses indicate the number of replicates for each treatment. Insect densities (mean \pm 1 SE
 574 insects/cm branch) were measured in November 2014.

575

HWA presence

		<i>0 years of HWA</i>	<i>2 years of HWA</i>	<i>4 years of HWA</i>
EHS presence	<i>0 years of EHS</i>	(12) Control EHS = 0 HWA = 0	(10) HWA-2 EHS = 0 HWA = $2.36 \pm$ 0.31	(13) HWA-4 EHS = 0 HWA = $1.74 \pm$ 0.20
	<i>2 years of EHS</i>	(9) EHS-2 EHS = $1.79 \pm$ 0.37 HWA = 0	(7) Both-2 EHS = $1.64 \pm$ 0.42 HWA = $1.11 \pm$ 0.22	(9) HWA \rightarrow Both EHS = $0.83 \pm$ 0.26 HWA = $1.29 \pm$ 0.27
	<i>4 years of EHS</i>	(9) EHS-4 EHS = $2.19 \pm$ 0.37 HWA = 0	(12) EHS \rightarrow Both EHS = $2.10 \pm$ 0.72 HWA = $1.37 \pm$ 0.33	(6) Both-4 EHS = $1.11 \pm$ 0.18 HWA = $1.21 \pm$ 0.24

576

577 **Table 2:** A) Mean amino acids ($\mu\text{g/g}$ dry tissue) from A) one-year needles and B) >1- year
 578 needles, by treatment and ranked in order of significance.

579 A.

Amino Acid	<i>F</i> -value	Rank	B-H <i>P</i> -value	Amino acid concentrations ($\mu\text{g g}^{-1}$ DM \pm SE)		
				0 years HWA	2 years HWA	4 years HWA
VAL	34.45	1	0.007	23.8 (1.3)	15.7 (1.7)	10.4 (1.1)
PRO	32.59	2	0.014	234.6 (22.3)	663.1 (71.1)	574.5 (48.5)
ILE	26.46	3	0.020	12.8 (0.8)	9.7 (2.2)	5.2 (0.7)
TRP	19.17	4	0.027	29.1 (1.7)	23.1 (1.5)	19.1 (1.3)
LYS	15.22	5	0.034	0.11 (0.011)	0.08(0.010)	0.05 (0.010)
THR	15.12	6	0.041	15.1 (1.0)	13.9 (0.8)	10.8 (1.0)
SER	13.48	7	0.048	202.1 (20.6)	207.9 (19.6)	143.4 (10.9)

580

581 B.

Amino Acid	<i>F</i> -value	Rank	B-H <i>P</i> -value	Amino acid concentrations ($\mu\text{g g}^{-1}$ DM \pm SE)		
				0 years HWA	2 years HWA	4 years HWA
PRO	21.27	1	0.007	199.4 (18.8)	417.5 (33.7)	370.1 (32.6)
VAL	14.02	2	0.014	18.5 (1.8)	13.1 (1.7)	9.3 (1.0)
TRP	13.15	3	0.021	18.3 (0.9)	16.1 (0.4)	14.5 (0.8)
ILE	11.28	4	0.029	13.5 (3.3)	7.3 (0.9)	4.7 (0.6)
THR	11.11	5	0.036	10.8 (0.8)	10.3 (1.2)	7.3 (0.8)
SER	5.65	6	0.043	140.8 (14.8)	152.2 (17.1)	120.6 (12.6)
LEU	4.56	7	0.050	3.4 (0.6)	2.6 (0.5)	2.0 (0.3)

582

583

584

585 **Figure Legends**

586 **Figure 1.** Ratio of (A, B) above- to below-ground biomass and (C, D) needle to wood
587 biomass in response to attack by hemlock woolly adelgid (HWA) (A, C) and elongate hemlock
588 scale (EHS) (B, D) following zero, two, or four years of infestation. Bars represent means \pm 1
589 SE. Letters indicate a significant difference among groups based on a post-hoc Tukey HSD test.

590 **Figure 2.** Mean \pm 1 SE rate of new foliage production (grams/day) in early spring
591 following zero, two, and four years of infestation by (A) hemlock woolly adelgid (HWA) and (B)
592 elongate hemlock scale (EHS). Letters indicate a significant difference among groups based on a
593 post-hoc Tukey test.

594 **Figure 3.** Mean \pm 1 SE of (A) photosynthetic rate, (B) percent nitrogen, (C) total starch
595 concentration, and (D) total amino acids across different tissue types (new flush, 1-yr needles,
596 >1-yr needles). Length of hemlock woolly adelgid (HWA) infestation spans zero years (light
597 orange), two years (medium orange), and four years (red). Letters indicate a significant
598 difference among groups based on post-hoc Tukey tests, while n.d. indicates a lack of data for
599 that tissue class and/or trait measurement.

600 **Figure 4.** Non-metric multidimensional scaling (NMDS) plots of amino acid profiles for
601 A) 1-year and B) >1-year needles following zero (yellow), two (orange), and four (red) years of
602 hemlock woolly adelgid (HWA) infestation. Symbols denote mean values; lines through symbols
603 denote standard errors.

604